UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

May 26, 2005

MEMORANDUM

Subject: Addendum of Efficacy Review for EPA File No. 74234-R, LMP-102™; DP Barcode: 301531

From: Tajah L. Blackburn, Ph.D., Microbiologist 5/26/05
Efficacy Evaluation Team
Product Science Branch
Antimicrobials Division (7510C)

Thru: Nancy Whyte, Acting Team Leader
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Product Science Branch
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To: Velma Noble PM 31/Jacqueline Campbell-McFarlane
Regulatory Management Branch I
Antimicrobials Division (7510C)

Applicant: Intralytix, Inc.
323 W. Camden Street
Baltimore, MD

Formulation from Label

Active Ingredient(s)	% by wt.
Listeria Specific Bacteriophage	0.00001%
Inert Ingredient(s)	99.99999%
Total	100.00000%

I BACKGROUND

This addendum is in response to the information requested from Intralytix, Inc., in support of the registration of LMP-102. The information provided by Intralytix, Inc., is detailed below, with relevant Agency comments.

II COMMENTS RELATED TO REQUESTED INFORMATION

Agency's Request
Clarification of the starting concentration *Listeria monocytogenes.*

Intralytix Response

The test system used in the non-food contact pathogen reduction test consisted of sterile glass coverslips inoculated with a mixture of three *Listeria monocytogenes* strains. Each coverslip was inoculated with 1 x 10^5 CFU of bacteria. **This concentration of the inoculum consistently resulted in recovery of 10^3 CFU from control samples, thereby permitting log reduction of ≥ 3 logs to be observed.** As stated in the "Assay Acceptance Criteria" section, "Organisms must be recoverable from the test surface at a concentration of 10^3 CFU per coverslip for the non-active control replicates"– and our using the above stated initial inoculum of 1 x 10^5 CFU of bacteria as the TEST CULTURE consistently met that criterion.

From a technical standpoint, the following 2-step procedure was employed to produce the targeted inoculum of 1 x 10^5 CFU/coverslip:

(i) A culture of each of the three *L. monocytogenes* test strains was grown separately until its OD_{600} reached 0.3-0.4, which equals approximately 1 x 10^9 CFU of *L. monocytogenes*/ml. Subsequently, each of the cultures was diluted 100-fold with sterile LB medium, in order to produce suspensions containing approximately 1 x 10^7 CFU of *L. monocytogenes*/ml.

(ii) In order to prepare the TEST CULTURE, equal volumes of each of the above suspensions were briefly and gently mixed together by vortexing. Subsequently, 10 µl of TEST CULTURE was used to contaminate each coverslip. Thus, the number of total CFU applied to each coverslip was 1 x 10^5 CFU, calculated as follows:

1 x 10^7 CFU/ml x (1 ml/1000 µl) x 10 µl = 1 x 10^5 CFU

The actual number of CFU/ml of the culture used to inoculate the coverslips was confirmed retrospectively, by plating the dilutions onto the MOX agar and determining the number of CFU/ml by a standard colony counting technique. The above methodology provided strong assurance that an inoculum of 1 x 10^5 CFU was used per coverslip, as stated in the report. However, as stipulated in the approved protocol, using a higher or lower bacterial inoculum would still be deemed acceptable, as long as, (i) three *L. monocytogenes* strains were mixed in essentially equal concentrations in the TEST CULTURE, and (ii) the number of bacteria recovered from the NON-ACTIVE CONTROL (NEGATIVE CONTROL) group was ≥

10^3 CFU per coverslip. As noted previously, the two criteria were consistently met during the 11/3/04 study.

Agency's Response

This response is sufficient. However, the 11/3/04 study date is inconsistent with submitted efficacy study. This is a possible typographical error. The PM should note that the Agency requires a starting inoculum larger than the required log reduction for quantitative tests (i.e., for a 3-log reduction, the starting inoculum is $\geq 10^4$).

Agency' Request

The Agency requested an explanation of the procedure used as the basis of the potency test, more specifically the absence of adequate agitation prior to spectrophotometer analysis. Furthermore, the Agency requested a protocol to explain the inevitability of phage acquired resistance of the preparation.

Intralytix Response

The POTENCY test has been developed by Intralytix as a means for ensuring the continuing potency of the LMP-102 preparation. The viability and titer of the active ingredient in LMP-102 (i.e., bacteriophages) are usually determined by a standard plaque-forming assay. In fact, that assay is used by Intralytix for each component monophage contained in LMP-102, as an integral part of each monophage's production process. However, after the monophages are combined in the cocktail (i.e., LMP-102), it is virtually impossible to determine accurately their titers in the mixture. For example, the various monophages in LMP-102 are grown in their own specific host cells, and their titers can be very different when they are determined with a different host cell (or they may not grow at all in a different host cell). Thus, our standard procedure is to determine the titer of each monophage before including it in LMP-102, and to infer the titer of LMP-102 as an arithmetic mean of all the monophages included in the preparation.

Although the above-described approach provides excellent assurance that all monophages are included in LMP-102 at their specified concentrations, it is not well-suited for determining the viability and potency of phages after they have been mixed together. Therefore, to address that latter issue, we have developed the current POTENCY protocol as means of ensuring the continuing potency of LMP-102. The protocol is based on the classical "Serial Dilutions" or "Dilution End-Points" assay which has been pioneered by Appelmans and Ellis and Delbruck in the 20s and 30s, and it has been historically used as an indicator for the presence of phage, and for enumerating bacteriophage particles and screening phages for their lytic activity against bacterial strains. [A] general description of the assay is outlined in (Carlson, K and E.S. Miller, 1994, Experimental protocols. Molecular Biology of Bacteriophage T4. *Ed* : J. D. Karam. Washington, DC, American Society of Microbiology) (relevant section of the above-cited reference was enclosed with Intralytix response letter). The assay is usually performed with aliquots of nutrient media in glass tubes, in which the host bacterium and various dilutions of phage-containing suspensions are mixed and incubated (usually without shaking) for a period of time (usually overnight). The tubes are then examined for bacterial lysis via the naked eye, and the clarity (or turbidity) of each tube is compared to the control tube

to which phage preparation has not been added. Clearing (reduction of turbidity) of the medium in the phage-treated tubes, compared to the turbidity of the medium in the phage-untreated control tubes, is indicative of the phage preparation's lytic activity (i.e., its potency).

During the development of our POTENCY test, we used the same "Serial Dilution" principle, but we modified the assay slightly in order to make the assay somewhat high-throughput, and to ensure that the results are more reproducible and quantitative, and less dependent on subjective interpretation of the technical personnel performing the assay. For example, we use microtiter plates instead of glass tubes during our assay. This enables us to analyze significantly larger number of samples at the same time, and to obtain our results by using ELISA microplate reader (instead of the naked eye). The latter approach eliminates the possible subjective interpretation of the results by technical personnel, since we have established clear-cut criteria for "positive" vs. "negative" OD_{600} values, thus eliminating the "completely cleared"– or "almost completely cleared"– type of guesswork during data collection and interpretation. The ELISA printouts can also be used as proof of actual OD_{600} readings which can be inserted into laboratory notebooks– an important consideration for all GLP laboratories that may use this approach in the future. **Our assay also avoids shaking the phage-bacteria mixture during incubation; i.e., the results are usually read without shaking the tubes, in order not to disturb bacterial debris that may accumulate at the bottom of the tubes (because the debris could be formed by both viable and phage-lysed bacterial cells, agitating the phage-bacteria mixture would complicate data analysis). Also, vigorous shaking of phages can deleteriously effect their viability and, subsequently, lead to misleading results. The POTENCY assay is well-standardized and has been validated by Intralytix in several experiments, including studies during which lots of LMP-102 that had lost potency were simulated by boiling LMP-102 samples for 5 minutes to inactivate the bacteriophages before being tested. In addition, the POTENCY test is always performed with appropriate controls handled exactly like the test samples (e.g., without shaking), which allows direct, side-by-side comparative analysis and ensures the robustness of the results.**

Agency's Response

The provided information is acceptable.

Agency's Request
The Agency requested the raw data that was used to generate the submitted graphs.

Intralytix's Response
Actual CFU counts used to generate Figure 6.2.1 (MRID No. 461693-04)– Corrected Version (Letter dated December 15, 2004)

Sample	Replicate	CFU counted	Dilution	Titer (CFU/ml)	Avg. Titer (CFU/ml) ±SD	Titer (Log CFU/ml)	Log Reduction
Dried org. control	1	123	-2	1.20E +04	1.4E +04 ±2.8E +03	4.2	NA
	2	160	-2	1.60E +04			
Non-active control	1	57	-2	5.70E +03	4.60E +03 ±1.5E +03	3.7	NA
	2	35	-2	3.50E +03			
Lot# 0103F240165	1	0	Undiluted	0.00E +00	0.00E +00 ±0	0	3.7
	2	0	Undiluted	0.00E +00			
Lot# 0103H260182	1	1	Undiluted	1.00E +00	4.00E +00 ±4.2E +00	0.6	3.1
	2	7	Undiluted	7.00E +00			
Neutralizer Group	1	86	-2	8.60E +03	8.6E +03 ±7.1 E +01	3.9	-0.3
	2	85	-2	8.50E +03			

NA= Not Applicable;
Under "Dilution", -2 indicates a 100-fold dilution
SD= Standard Deviation

Table 2 Actual CFU counts used to generate Figure 6.2.2 (MRID No. 461693-04)– Corrected version (Letter dated December 15, 2004)

Sample	Replicate	CFU counted	Dilution	Titer (CFU/ml)	Avg. Titer (CFU/ml) ±SD	Titer (Log CFU/ml)	Log Reduction
Dried org. control	1	74	-2	7.40E +03	6.9E +03 ±5.0E+02	3.8	NA
	2	70	-2	7.00E +03			
	3	64	-2	6.40E +03			
Non-active control	1	46	-2	4.60E +03	4.5E +03 ±1.2 E +03	3.7	NA
	2	56	-2	5.60E +03			
	3	33	-2	3.30E +03			

Lot# 0103F240165	1	0	Undiluted	0.00E +00	0.0E +0 ±00	0	3.7
	2	0	Undiluted	0.00E +00			
	3	0	Undiluted	0.00E +00			
Lot# 0103H260182	1	2	Undiluted	2.00E +00	1.0E +00 ±1.0 E +0	0	3.7
	2	1	Undiluted	1.00E +00			
	3	0	Undiluted	0.00E +00			
Lot# 0103J270152	1	0	Undiluted	0.00E +00	0.3E +00 ±0.6 E +00	0	4.1
	2	0	Undiluted	0.00E +00			
	3	0	Undiluted	1.00E +00			
Neutralizer group	1	208	-1	2.10E +03	1.9 E +03 ±1.5 E +02	3.3	0.4
	2	184	-1	1.80E +03			
	3	188	-1	1.90E +03			

NA= Not Applicable;
Under "Dilution", -2 indicates a 100-fold dilution
SD= Standard Deviation

Agency's Response
The requested information is acceptable.

Additional Intralytix's Comments
Please note that Intralytix uses two numbering systems to identify phage production batches. The first system is a shorthand numbering system used to label tubes, plates, vials, etc., which consist of the date the batch was made and a letter designation if more than one batch was produced on the same day. The second system is a commercial numbering system used for phage products that go outside of Intralytix. This commercial system uses the same shorthand numbering system described above, but it contains additional tracking information. During all of Intralytix's internal studies, the two numbering systems can be, and often are, used interchangeably. Thus, the batches of LMP-102 used in the experiments described in the original internal report were identified by either of these two systems/lot numbers:

Lot#6/24/03 is the same as Lot# 0103F240165
Lot#8/26/03B is the same as Lot# 0103H260182
Lot#10/27/03 is the same as Lot# 0103J270152

Agency's Request
Per the letter emailed from Eliot Harrison, justification for submission of non-GLP study has been provided to the Agency. According to the submitted letter, the applicant's position is that "even though the study was not certified as GLP compliant, we believe the study is valid and reproducible particularly since many of the key GLP elements." Several elements essential to GLP are required for submission of non-GLP study. The missing elements with appropriate Intralytix's responses are addressed below:

(a) **Missing Item**: Experimental start date.
Intralytix's Response: October 15, 2003

(b) **Missing Item:** Experimental termination date.
Intralytix's Response: October 29, 2003

(c) **Missing Item**: Study completion date.
Intralytix's Response: November 3, 2003

(d) **Missing Item:** Study initiation date.
Intralytix's Response: October 6, 2003

(e) **Missing Item**: The statistical methods employed for analyzing data [40 CFR §160.185(j)(3)];
Intralytix's Response: As described in our report of 11/3/04, 5 minutes of exposure to the applied LMP-102 preparation produced a ≥3 log reduction in the number of *Listeria monocytogenes* colony-forming units (CFU). **Due to this high degree of reduction, statistical methods were not employed to analyze the original data.** We have revised the tables previously submitted as part of our response dated December 1, 2004, to include the standard deviations of the mean titers. The standard deviations were calculated from the data using the column statistics function of Prism for Windows V 4.00. GraphPad Software, Inc. (www.graphpad.com). We have also corrected a clerical error in the labeling of the Tables 1 and 2, which now correctly indicate correspondence to figures 6.2.1 and 6.2.2 in the December 29, 2003 submission. No statistical analyses were applied to the data contained in Tables 6.1.1 and 6.1.2 in the December 29, 2003 submission. Please note that the tables submitted in the response dated December 3, 2003 contained several minor clerical errors that have now been corrected.

(f) **Additional Intralytix's Comments**: By way of background, Intralytix currently does not maintain a formal GLP laboratory; rather, it operates a modern R & D facility at the Columbus Center in downtown Baltimore. However, all of Intralytix's studies are performed with the same level of scientific rigor that is expected of high-level academic and small biotech company laboratories, and the information you requested regarding the above-referenced study's start date, termination date, etc., was recorded and is available. The project's final report was submitted to the EPA on November 3, 2004, and it was prepared– by Intralytix– based on results of four separate studies (efficacy studies #10 and #12 plus one potency study and one combined potency/stability study). That report did not specify the exact starting, termination, etc., dates of each component study; rather, it indicated the time period during which all of the studies comprising the 11/3/04 report were conducted.
Agency's Response: Data and information provided as requested. Tables are listed below.

(g) **Missing Item**: Signatures were omitted from page 5 of 27 of the submitted efficacy study (MRID No. 461693-04); The signed and dated reports of each of the individual scientist or other professional involved in the study, including each person who at the request or direction of the testing facility or sponsor, conducted an analysis of evaluation of data or specimens from the study after data generation was completed [40 CFR §160.185 (j)(12)].
Intralytix's Response: A signed copy of page 5 of the study report is attached [to this addendum]. The signatures are those of the Study Director and the Chief Scientific Officer of Intralytix.
Agency's Response: Information provided as requested (see Attachment I).

(h) **Missing Item**: The statement prepared and signed by the quality assurance unit [40 CFR §160.185 (j)(14)].
Intralytix's Response: As explained above, Intralytix currently does not have formal GLP laboratory with a separate quality assurance unit. Instead, the company uses a rigorous system of multi-layer data confirmation and analysis, which consists of at least three different individuals reviewing the study's design and outcomes. In this context, as evidenced by the attached hard copies of relevant studies, the study's design and data were reviewed by (i) the technical personnel directly involved in performing the study, (ii) the study director, and (iii) Intralytix's Chief Scientist. We believe that this level of review and record keeping provides a strong assurance of the experiments' rigor and that it provides the necessary quality assurance for a non-GLP laboratory.
Agency's Response: Explanation is acceptable as outlined. However, the Agency requires that efficacy studies be conducted under GLP guidelines.

(i) **Missing Item**: The final report should be signed and dated by the study director [40 CFR §160.185 (j)(14)(b)].
Intralytix's Response: A signed copy was included in the original submission and is attached. Please note that I [Eliot Harrison] signed on behalf of the study director.
Agency's Response: Information provided as requested (see Attachment II).

III ADDITIONAL QUESTIONS POSED TO INTRALYTIX

Question: The first step of bacteriophage infection is adsorption, or the attachment of the virus to the bacterial surface. Adsorption has two primary stages, a reversible stage and an irreversible stage. In the reversible stage the tail fibers at the tip of the tail attach to the bacterial surface. Bacteriophages attach to specific outer membrane proteins, which surrounds the rigid peptidoglycan layer of the bacterial cell wall. There may also be other bacteriophage receptors that facilitate the process of attaching to the bacterial cell. Phage attachment is reversible until the second stage of adsorption, in which the short tail fibers attach and the phage becomes permanently attached. **What is the timeline of the bacteriophage attachment process, as it relates to the specified contact time on the proposed label?**

Response from Intralytix: A time period of 20-40 seconds is needed for irreversible attachment to occur; this is well within the 5 minute contact time.

Information provided by Arvind A. Bhagwat, Ph.D. (USDA)–External scientist
Laboratory conditions provide an ideal environment for irreversible attachment in 3-5 minutes. Considering that this product is for use in plant setting this attachment maybe be longer than the cited 3-5 minutes. However, no literature is available to confirm or deny this hypothesis.

Agency's Response
In the absence of data regarding the irreversible attachment of the bacteriophages in LMP-102, the information provided by Intralytix is sufficient to support a 5-minute contact time. For future reference, contact times less than 5 minutes will require additional data to document the irreversible attachment stage before such reduced contact times are accepted.

***Question*: The listed contact times for the laboratory study and field study are inconsistent. What is the effective contact time for LMP-102?**
Response from Intralytix: Per the laboratory studies, the contact time is five minutes. The Field Tests should not be referenced in support of product registration.

Agency's Response. The Field Tests do indeed increase the Agency's skepticism of the true efficacy of LMP-102. For registration purposes the Field Tests are not required, but will remain as a reference in the data package.

***Question*: What type of Quality Assurance program is utilized to test and address bacteriophage acquired resistance?**
Response from Intralytix: Measures are currently in place to address resistant bacteriophages.

Agency's Response
These measures were not detailed. The measures that will be used to test and address bacteriophage acquired resistance need to be fully described to the Agency.

IV CONCLUSIONS

1. Intralytix, Inc., has respectfully addressed the deficiencies and questions posed in the original product review (dated October 29, 2004). As mentioned in the previous review and cited in the current, the study was not conducted in accordance to GLP guidelines and standards. Since the data was not generated using GLP guidelines, the Agency cannot accept this study to support the registration of the LMP-102. In addition, the Agency believes that the protocols and questions generated during this review should be presented to the Scientific Advisory Panel (SAP) for further review and comment. Further insight generated from these scientists with expertise in bacteriophage studies will be most advantageous as the Agency advances in accepting novel technology. The Agency will commence assembling a panel of experts for a letter SAP and will provide the applicant with a timeline for their review.

V RECOMMENDATIONS

1. Intralytix, Inc., must conduct a new GLP study and submit the results for review to the Agency. However, they may want to await the recommendations from the SAP prior to conducting this study.

2. Intralytix, Inc. must provide the Agency with a standard operating procedure (SOP) to address the inevitability of attenuated bacteriophage effectiveness due to bacteria resistance. This plan will be presented to the SAP for review and comment.